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L1	immuno-PCR or immuno-aRNA	57	L1

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L3: Entry 1 of 1 File: USPT Sep 24, 1996

DOCUMENT-IDENTIFIER: US 5559000 A
TITLE: Encoded reaction cassette

Brief Summary Text (7):
In instances in which one can obtain antibodies to the reaction product or substrate, an immuno-PCR assay may be constructed and employed as a detection system. (T. Sano et al., Science (1992): vol. 258, pages 120-122). An immuno-PCR assay is similar to an enzyme immunoassay except that the enzyme-antibody conjugate is replaced by an antibody conjugated to a PCR amplifiable polynucleotide strand. Immuno-PCR assays are highly sensitive. However, at very low levels of antigen, the immuno-PCR assay is limited by non-specific binding of the antibody-polynucleotide conjugate.

Detailed Description Text (17):
An alternative to analyzing the PCR products on agarose gel, which can become laborious when libraries of catalysts are being screened, one can simply add to the reaction mixture a fluorescent probe that undergoes fluorescence enhancement upon intercalation into the DNA. The insert in FIG. 4 shows a photograph taken under UV light (254 nm) of the reaction media in the presence of the YOYO-1 . The first well corresponds to the experiment in lane 6, the second well to the experiment in lane 8, and the third to the probe in buffered solution without any additives. The greatest fluorescence enhancement is in the first well which contains the amplified DNA. The second well shows a background fluorescence resulting from the interaction of the probe with the primers. As expected, the third well does not show any detectable fluorescence. Another advantage of the YOYO-1 probe is that the amount of the PCR product (which should be directly related to the efficiency of the enzyme cleavage) can be quantified, e.g., M. Ogura et al., Biotechniques (1994): vol. 16, pages 1032-1033.

Detailed Description Text (25):
The chemicals were purchased from Novabiochem and Aldrich for peptide synthesis, from Millipore for DNA synthesis, and from Promega for the PCR experiments. The YOYO-1 probe was purchased from Molecular Probes Inc. The solvents were purchased from Fisher or Baxter (water content <0.001%). For synthesis of the linkers, the chemicals were purchased from Aldrich, and were used without any further purification.

Detailed Description Text (32):
Fluorescence Assay. After the PCR, the reaction supernatant (25 ml) was transferred to a 96-well ELISA plate and diluted to 250 ml with dH.sub.2 O (175 ml) and methanol (50 ml). The probe (1 ml, YOYO-1) was added to this media, and the results were analyzed under UV light (254 nm).

End of Result Set

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L5: Entry 5 of 5 File: USPT Sep 9, 1997

DOCUMENT-IDENTIFIER: US 5665539 A
TITLE: Immuno-polymerase chain reaction system for antigen detection

Detailed Description Text (3):
There are several novel features involved in this invention. First, a nucleic acid sequence is used as the marker for detection of antigen. Second, antibody-linker-biotinylated marker conjugates are used to attach a marker molecule to an antigen. Third, enzymatic steps such as a polymerase chain reaction are used to amplify signals for detection of specific antigens. Fourth, due to the specificity and efficiency of nucleic acid amplification, the detection sensitivity of the immuno-PCR technology is superior to that of any existing antigen detection system and the method is, in principle, able to detect a single antigen molecule. No method of such sensitivity is currently available. Fifth, a wider variety of antigens can be detected by the immuno-PCR than by other currently available antigen detection systems.

Detailed Description Text (4):
Briefly, in the current invention, a linker molecule with bispecific binding affinity for nucleic acids and antibodies is used to attach a DNA, RNA, DNA/RNA hybrid, or their fragment, analogue or derivative molecule used as a marker, specifically to an antigen-antibody complex, resulting in the formation of a specific antigen-antibody-linker-DNA conjugate. A segment of the attached marker is amplified enzymatically (such as by a polymerase chain reaction with appropriate primers). The presence of specific products of polymerase chain reaction or other amplification methods demonstrates that marker molecules are attached specifically to antigen-antibody complexes and in turn, this indicates the presence of antigen.

Detailed Description Text (6):
In the current invention, a streptavidin-protein A chimera or any other linker that possesses tight and specific binding affinity both for biotin and immunoglobulin G was used to attach a biotinylated marker specifically to antigen-monoclonal antibody complexes that had been immobilized on microtiter plate wells. Next, a segment of the attached marker was amplified by PCR. Analysis of the PCR products by agarose gel electrophoresis after staining with ethidium bromide allowed as few as 580 antigen molecules (9.6.times.10.sup.-22 moles) to be readily and reproducibly detected. Direct comparison with enzyme-linked immunosorbent assays (ELISA) with the use of a chimera-biotinylated alkaline phosphatase conjugate demonstrated that approximately 10.sup.5 times enhancement in antigen detection sensitivity was obtained with the use of immuno-PCR. Given the enormous amplification capability and specificity of PCR, the current immuno-PCR technology has a sensitivity greater than that of any existing antigen detection system and, in principle, is sensitive enough to be applied to the detection of single antigen molecules.

Detailed Description Text (7):
One mode of the immuno-PCR technology of the current invention, in which a specific antibody-DNA conjugate is used to detect antigens, utilizes immobilization of various amounts of an antigen on the surface of microtiter plate wells. For initial testing, bovine serum albumin (BSA) was used as the antigen because of the availability of both the pure protein and monoclonal antibodies against it. The detection procedure used was similar to conventional enzyme-linked immunosorbent assays (ELISA). Instead of an enzyme-conjugated secondary antibody directed against the primary antibody, as in typical ELISA, a biotinylated linear plasmid DNA (pUC19), conjugated to the streptavidin-protein A chimera, was targeted to the antigen-antibody complexes. A segment of the attached marker was amplified by PCR with appropriate primers and the resulting PCR products were analyzed by agarose gel electrophoresis after staining

with the ethidium bromide.

Detailed Description Text (8):
The concept of immuno-PCR of the current invention is shown schematically in FIG. 1. By using linker molecule X which has bispecific binding affinity both for the marker for antibody, a molecule used as a marker can be specifically attached to an antibody-antigen complex. Marker molecules are typically DNA, RNA, DNA-RNA hybrids, their derivatives, fragments, segments or analogues. The attached marker allows the amplification of its segment(s) by PCR with appropriate primers. The enormous amplification capability and specificity of PCR allows the production of large amounts of specific DNA segments as PCR's products. These products can be detected by various methods known and used in molecular biology such as, for example, by agarose gel electrophoresis. The presence of specific PCR products demonstrates that marker molecules are attached to antigen-antibody complexes, indicating the presence of antigen. In addition, the quantitation of PCR products also provides the estimation of the number of antigens (epitope).

Detailed Description Text (17):
A specific marker molecule was used as a marker in the conjugated DNA-linker-antibody-antigen complex. The marker molecule may be DNA, RNA, DNA-RNA hybrid, fragment, segment, their derivative and analogue. The marker DNA, as used in this invention, serves as the entity recognizable and amplifiable by PCR. For the purposes of this invention, the marker DNA is biotinylated. Biotinylated DNA is easily conjugated to the streptavidin moiety of the streptavidin-protein A chimera allowing a specific binding of biotinylated DNA to the chimera.

Detailed Description Text (47):
Detection of a label which has been incorporated directly into marker DNA is performed by methods known in the art. Many labels, which can be incorporated specifically into DNA, are available. They include fluorescent dyes such as for example, ethidium bromide and ethidium homodimer, which are frequently used in molecular biology. Another way is to attach a hapten such as for example, biotin and FITC, to the marker DNA, followed by the detection of the label by using, for example, antibodies against the label.

Detailed Description Text (49):
Detection of Antigen Immobilized on Microtiter Plate by Immuno-PCR

Detailed Description Text (52):
Because of the availability of pure antigen and of monoclonal antibodies against it, to develop and to test the immuno-PCR method of this invention, bovine serum albumin (BSA) was used as the antigen. The procedures for detecting BSA immobilized on a microtiter plate by immuno-PCR are described below. The detection method and assays for other antigens are run under the same or slightly modified conditions.

Detailed Description Text (55):
Immuno-PCR technology using a specific DNA molecule as the marker can reliably detect very small amounts of antigen with sufficient reproducibility. The sensitivity of immuno-PCR is superior to that of any existing antigen detection system. Therefore, this technology is particularly useful for detecting rare antigen molecules in biological samples. The extremely high sensitivity of immuno-PCR enables this method to detect single antigen molecules; no current method allows detection with such a degree of sensitivity. The sensitivity of immuno-PCR can be controlled by varying one or more key factors. This suggests that immuno-PCR technology can be used on a wide variety of biological samples.

Detailed Description Text (63):
In principle, the immuno-PCR technology can be applied to detection of single molecules. No method is currently available which would have the same degree of sensitivity. In addition, the extremely high sensitivity of the immuno-PCR considerably reduces the amounts of antibodies required resulting in reduced assay costs. This is particularly useful when large amounts of specific antibodies are not available.

Detailed Description Text (64):

. The sensitivity of immuno-PCR can be controlled by varying one or more key factors, such as the number of amplification cycles and the detection methods for PCR products. This characteristic allows immuno-PCR technology to be applied to a wide range of biological samples, in which the number of antigens varies considerably.

Detailed Description Text (65):
Another advantage of immuno-PCR technology is its simplicity. Immuno-PCR does not include any complex procedures. Thus it allows the development of fully automated assay systems without a loss of sensitivity. This characteristic offers a great promise for applications in clinical diagnostics.

Detailed Description Text (66):
Although DNA is used as a marker in the experiments described above, other nucleic acid molecules such as RNA can be used as the marker upon biotinylation.

Detailed Description Text (68):
Using a similar configuration, any antigen molecule, which is efficiently separated from unbound antibody and unbound chimera-nucleic acid conjugates, can be detected by immuno-PCR without the need for modification of the basic procedure. Such antigen molecules include:

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L5: Entry 1 of 5 File: USPT Mar 12, 2002

DOCUMENT-IDENTIFIER: US 6355431 B1
TITLE: Detection of nucleic acid amplification reactions using bead arrays

Brief Summary Text (8):
The polymerase chain reaction (PCR) is widely used and described, and involves the use of primer extension combined with thermal cycling to amplify a target sequence; see U.S. Pat. Nos. 4,683,195 and 4,683,202, and PCR Essential Data, J. W. Wiley & sons, Ed. C. R. Newton, 1995, all of which are incorporated by reference. In addition, there are a number of variations of PCR which also find use in the invention, including "quantitative competitive PCR" or "QC-PCR", "arbitrarily primed PCR" or "AP-PCR", "immuno-PCR", "Alu-PCR", "PCR single strand conformational polymorphism" or "PCR-SSCP", allelic PCR (see Newton et al. Nucl. Acid Res. 17:2503 91989); "reverse transcriptase PCR" or "RT-PCR", "biotin capture PCR", "vectorette PCR". "panhandle PCR", and "PCR select cDNA subtraction", among others.

Brief Summary Text (11):
Cycling probe technology (CPT) is a nucleic acid detection system based on signal or probe amplification rather than target amplification, such as is done in polymerase chain reactions (PCR). Cycling probe technology relies on a molar excess of labeled probe which contains a scissile linkage of RNA. Upon hybridization of the probe to the target, the resulting hybrid contains a portion of RNA:DNA. This area of RNA:DNA duplex is recognized by RNAseH and the RNA is excised, resulting in cleavage of the probe. The probe now consists of two smaller sequences which may be released, thus leaving the target intact for repeated rounds of the reaction. The unreacted probe is removed and the label is then detected. CPT is generally described in U.S. Pat. Nos. 5,011,769, 5,403,711, 5,660,988, and 4,876,187, and PCT published applications WO 95/05480, WO 95/1416, and WO 95/00667, all of which are specifically incorporated herein by reference.

Brief Summary Text (25):
In addition, wherein the first target sequence is an RNA target sequence, the first primer nucleic acid is a DNA primer comprising an RNA polymerase promoter, the first enzyme is a reverse-transcriptase that extends the first primer to form a first newly synthesized DNA strand, the method further comprises the addition of a second enzyme comprising an RNA degrading enzyme that degrades the first target sequence, the addition of a third primer that hybridizes to the first newly synthesized DNA strand, the addition of a third enzyme comprising a DNA polymerase that extends the third primer to form a second newly synthesized DNA strand, to form a newly synthesized DNA hybrid, the addition of a fourth enzyme comprising an RNA polymerase that recognizes the RNA polymerase promoter and generates at least one newly synthesized RNA strand from the DNA hybrid, such that nucleic acid sequence-based amplification (NASBA) occurs.

Brief Summary Text (27):
An additional aspect of the invention is a method for detecting a target nucleic acid sequence comprising hybridizing a first primer to a first target sequence to form a first hybridization complex, contacting the first hybridization complex with a first enzyme to extend the first primer to form a first newly synthesized strand and form a nucleic acid hybrid that comprises an RNA polymerase promoter, contacting the hybrid with an RNA polymerase that recognizes the RNA polymerase promoter and generates at least one newly synthesized RNA strand, contacting the newly synthesized RNA strand with an array comprising a substrate with a surface comprising discrete sites and a population of microspheres comprising at least a first subpopulation comprising a first capture probe; such that the first capture probe and the modified primer form an assay complex; wherein the microspheres are distributed on the surface and detecting the presence of the newly synthesized RNA strand.

Brief Summary Text (28):
In addition, when the target nucleic acid sequence is an RNA sequence, and prior to hybridizing a first primer to a first target sequence to form a first hybridization complex, method comprises hybridizing a second primer comprising an RNA polymerase promoter sequence to the RNA sequence to form a second hybridization complex, contacting the second hybridization complex with a second enzyme to extend the second primer to form a second newly synthesized strand and form a nucleic acid hybrid; and degrading the RNA sequence to leave the second newly synthesized strand as the first target sequence. In one aspect of the invention the degrading is done by the addition of an RNA degrading enzyme. In an additional aspect of the invention the degrading is done by RNA degrading activity of reverse transcriptase.

Brief Summary Text (29):
In addition, when the target nucleic acid sequence is a DNA sequence, and prior to hybridizing a first primer to a first target sequence to form a first hybridization complex, the method comprises hybridizing a second primer comprising an RNA polymerase promoter sequence to the DNA sequence to form a second hybridization complex, contacting the second hybridization complex with a second enzyme to extend the second primer to form a second newly synthesized strand and form a nucleic acid hybrid, and denaturing the nucleic acid hybrid such that the second newly synthesized strand is the first target sequence.

Brief Summary Text (34):
In an additional aspect of the invention, is a kit for the detection of a NASBA reaction wherein the first enzyme is a reverse transcriptase, and the kit comprises a second enzyme comprising an RNA degrading enzyme, a third primer, a third enzyme comprising a DNA polymerase and a fourth enzyme comprising an RNA polymerase.

Detailed Description Text (4):
Accordingly, the present invention provides compositions and methods for detecting the presence or absence of target nucleic acid sequences in a sample. As will be appreciated by those in the art, the sample solution may comprise any number of things, including, but not limited to, bodily fluids (including, but not limited to, blood, urine, serum, lymph, saliva, anal and vaginal secretions, perspiration and semen, of virtually any organism, with mammalian samples being preferred and human samples being particularly preferred); environmental samples (including, but not limited to, air, agricultural, water and soil samples); biological warfare agent samples; research samples; purified samples, such as purified genomic DNA, RNA, proteins, etc.; raw samples (bacteria, virus, genomic DNA, etc.; As will be appreciated by those in the art, virtually any experimental manipulation may have been done on the sample.

Detailed Description Text (8):
Particularly preferred are peptide nucleic acids (PNA) which includes peptide nucleic acid analogs. These backbones are substantially non-ionic under neutral conditions, in contrast to the highly charged phosphodiester backbone of naturally occurring nucleic acids. This results in two advantages. First, the PNA backbone exhibits improved hybridization kinetics. PNAs have larger changes in the melting temperature (Tm) for mismatched versus perfectly matched basepairs. DNA and RNA typically exhibit a 2-4.degree. C. drop in Tm for an internal mismatch. With the non-ionic PNA backbone, the drop is closer to 7-9.degree. C. This allows for better detection of mismatches. Similarly, due to their non-ionic nature, hybridization of the bases attached to these backbones is relatively insensitive to salt concentration.

Detailed Description Text (9):
The nucleic acids may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. The nucleic acid may be DNA, both genomic and cDNA, RNA or a hybrid, where the nucleic acid contains any combination of deoxyribo- and ribo-nucleotides, and any combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xathanine hypoxathanine, isocytosine, isoguanine, etc. A preferred embodiment utilizes isocytosine and isoguanine in nucleic acids designed to be complementary to other probes, rather than target sequences, as this reduces non-specific hybridization, as is generally described in U.S. Pat. No. 5,681,702. As used herein, the term

"nucleoside" includes nucleotides as well as nucleoside and nucleotide analogs, and modified nucleosides such as amino modified nucleosides. In addition, "nucleoside" includes non-naturally occuring analog structures. Thus for example the individual units of a peptide nucleic acid, each containing a base, are referred to herein as a nucleoside.

Detailed Description Text (10):
The compositions and methods of the invention are directed to the detection of target sequences. The term "target sequence" or "target nucleic acid" or grammatical equivalents herein means a nucleic acid sequence on a single strand of nucleic acid. The target sequence may be a portion of a gene, a regulatory sequence, genomic DNA, cDNA, RNA including mRNA and rRNA, or others. As is outlined herein, the target sequence may be a target sequence from a sample, or a secondary target such as a product of a reaction such as a detection sequence from an invasive cleavage reaction, a ligated probe from an OLA reaction, an extended probe from a PCR reaction, etc. Generally, as outlined herein, a target sequence from a sample is amplified to produce a secondary target that is detected, as outlined herein. Alternatively, an amplification step is done using a signal probe that is amplified, again producing a secondary target that is detected. The target sequence may be any length, with the understanding that longer sequences are more specific. As will be appreciated by those in the art, the complementary target sequence may take many forms. For example, it may be contained within a larger nucleic acid sequence, i.e. all or part of a gene or mRNA, a restriction fragment of a plasmid or genomic DNA, among others. As is outlined more fully below, probes are made to hybridize to target sequences to determine the presence or absence of the target sequence in a sample. Generally speaking, this term will be understood by those skilled in the art. The target sequence may also be comprised of different target domains; for example, in "sandwich" type assays as outlined below, a first target domain of the sample target sequence may hybridize to a capture probe or a portion of capture extender probe, a second target domain may hybridize to a portion of an amplifier probe, a label probe, or a different capture or capture extender probe, etc. In addition, the target domains may be adjacent (i.e. contiguous) or separated. For example, when LCR techniques are used, a first primer may hybridize to a first target domain and a second primer may hybridize to a second target domain; either the domains are adjacent, or they may be separated by one or more nucleotides, coupled with the use of a polymerase and dNTPs, as is more fully outlined below. The terms "first" and "second" are not meant to confer an orientation of the sequences with respect to the 5'-3' orientation of the target sequence. For example, assuming a 5'-3' orientation of the complementary target sequence, the first target domain may be located either 5' to the second domain, or 3' to the second domain.

Detailed Description Text (25):
In a preferred embodiment, the target amplification technique is PCR. The polymerase chain reaction (PCR) is widely us ed and described, and involves the use of primer extension combined with thermal cycling to amplify a target sequence; see U.S. Pat. Nos. 4,653,195 and 4,683,202, and PCR Essential Data, J. W. Wiley & sons, Ed. C. R. Newton, 1995, all of which are incorporated by reference. In addition, there are a number of variations of PCR which also find use in the invention, including "quantitative competitive PCR" or "QC-PCR", "arbitrarily primed PCR" or "AP-PCR", "immuno-PCR", "Alu-PCR", "PCR single strand conformational polymorphism" or "PC R-SSCP", "reverse transcriptase PCR" or "RT-PCR", "biotin capture PCR", "vectorette PCR", "panhandle PCR", and "PCR select cDNA subtraction", "allele-specific PCR", among others.

Detailed Description Text (38):
In a preferred embodiment, the target amplification technique is nucleic acid sequence based amplification (NASBA). NASBA is generally described in U.S. Pat. No. 5,409,818; Sooknanan et al., Nucleic Acid Sequence-Based Amplification, Ch. 12 (pp. 261-285) of Molecular Methods for Virus Detection, Academic Press, 1995; and "Profiting from Gene-based Diagnostics", CTB International Publishing Inc., N.J., 1996, all of which are incorporated by reference. NASBA is very similar to both TMA and QBR. Transcription mediated amplification (TMA) is generally described in U.S. Pat. Nos. 5,399,491, 5,888,779, 5,705,365, 5,710,029, all of which are incorporated by reference. The main difference between NASBA and TMA is that NASBA utilizes the addition of RNAse H to effect RNA degradation, and TMA relies on inherent RNAse H

activity of the reverse transcriptase.

Detailed Description Text (39):
In general, these techniques may be described as follows. A single stranded target nucleic acid, usually an RNA target sequence (sometimes referred to herein as "the first target sequence" or "the first template"), is contacted with a first primer, generally referred to herein as a "NASBA primer" (although "TMA primer" is also suitable). Starting with a DNA target sequence is described below. These primers generally have a length of 25-100 nucleotides, with NASBA primers of approximately 50-75 nucleotides being preferred. The first primer is preferably a DNA primer that has at its 3' end a sequence that is substantially complementary to the 3' end of the first template. The first primer also has an RNA polymerase promoter at its 5' end (or its complement (antisense), depending on the configuration of the system). The first primer is then hybridized to the first template to form a first hybridization complex. The reaction mixture also includes a reverse transcriptase enzyme (an "NASBA reverse transcriptase") and a mixture of the four dNTPs, such that the first NASBA primer is modified, i.e. extended, to form a modified first primer, comprising a hybridization complex of RNA (the first template) and DNA (the newly synthesized strand).

Detailed Description Text (40):
By "reverse transcriptase" or "RNA-directed DNA polymerase" herein is meant an enzyme capable of synthesizing DNA from a DNA primer and an RNA template. Suitable RNA-directed DNA polymerases include, but are not limited to, avian myloblastosis virus reverse transcriptase ("AMV RT") and the Moloney murine leukemia virus RT. When the amplification reaction is TMA, the reverse transcriptase enzyme further comprises a RNA degrading activity as outlined below.

Detailed Description Text (41):
In addition to the components listed above, the NASBA reaction also includes an RNA degrading enzyme, also sometimes referred to herein as a ribonuclease, that will hydrolyze RNA of an RNA:DNA hybrid without hydrolyzing single- or double-stranded RNA or DNA. Suitable ribonucleases include, but are not limited to, RNase H from E. coli and calf thymus.

Detailed Description Text (42):
The ribonuclease activity degrades the first RNA template in the hybridization complex, resulting in a disassociation of the hybridization complex leaving a first single stranded newly synthesized DNA strand, sometimes referred to herein as "the second template".

Detailed Description Text (43):
In addition, the NASBA reaction also includes a second NASBA primer, generally comprising DNA (although as for all the probes herein, including primers, nucleic acid analogs may also be used). This second NASBA primer has a sequence at its 3' end that is substantially complementary to the 3' end of the second template, and also contains an antisense sequence for a functional promoter and the antisense sequence of a transcription initiation site. Thus, this primer sequence, when used as a template for synthesis of the third DNA template, contains sufficient information to allow specific and efficient binding of an RNA polymerase and initiation of transcription at the desired site. Preferred embodiments utilizes the antisense promoter and transcription initiation site are that of the T7 RNA polymerase, although other RNA polymerase promoters and initiation sites can be used as well, as outlined below.

Detailed Description Text (45):
Finally, the inclusion of an RNA polymerase and the required four ribonucleoside triphosphates (ribonucleotides or NTPs) results in the synthesis of an RNA strand (a third newly synthesized strand that is essentially the same as the first template). The RNA polymerase, sometimes referred to herein as a "DNA-directed RNA polymerase", recognizes the promoter and specifically initiates RNA synthesis at the initiation site. In addition, the RNA polymerase preferably synthesizes several copies of RNA per DNA duplex. Preferred RNA polymerases include, but are not limited to, T7 RNA polymerase, and other bacteriophage RNA polymerases including those of phage T3, phage .phi.II, Salmonella phage sp6, or Pseudomonase phage gh-1.

Detailed Description Text (46):

In some embodiments, TMA and NASBA are used with starting DNA target sequences. In this embodiment, it is necessary to utilize the first primer comprising the RNA polymerase promoter and a DNA polymerase enzyme to generate a double stranded DNA hybrid with the newly synthesized strand comprising the promoter sequence. The hybrid is then denatured and the second primer added.

Detailed Description Text (47):
Accordingly, the NASBA reaction requires, in no particular order, a first NASBA primer, a second NASBA primer comprising an antisense sequence of an RNA polymerase promoter, an RNA polymerase that recognizes the promoter, a reverse transcriptase, a DNA polymerase, an RNA degrading enzyme, NTPs and dNTPs, in addition to the detection components outlined below.

Detailed Description Text (48):
These components result in a single starting RNA template generating a single DNA duplex; however, since this DNA duplex results in the creation of multiple RNA strands, which can then be used to initiate the reaction again, amplification proceeds rapidly.

Detailed Description Text (49):
Accordingly, the TMA reaction requires, in no particular order, a first TMA primer, a second TMA primer comprising an antisense sequence of an RNA polymerase promoter, an RNA polymerase that recognizes the promoter, a reverse transcriptase with RNA degrading activity, a DNA polymerase, NTPs and dNTPs, in addition to the detection components outlined below.

Detailed Description Text (50):
These components result in a single starting RNA template generating a single DNA duplex; however, since this DNA duplex results in the creation of multiple RNA strands, which can then be used to initiate the reaction again, amplification proceeds rapidly.

Detailed Description Text (51):
As outlined herein, the detection of the newly synthesized strands can proceed in several ways. Direct detection can be done when the newly synthesized strands comprise detectable labels, either by incorporation into the primers or by incorporation of modified labelled nucleotides into the growing strand. Alternatively, as is more fully outlined below, indirect detection of unlabelled strands (which now serve as "targets" in the detection mode) can occur using a variety of sandwich assay configurations. As will be appreciated by those in the art, any of the newly synthesized strands can serve as the "target" for form an assay complex on a surface with a capture probe. In NASBA and TMA, it is preferable to utilize the newly formed RNA strands as the target, as this is where significant amplification occurs.

Detailed Description Text (120):
In a preferred embodiment, the scissile linkage comprises RNA. This system, previously described in as outlined above, is based on the fact that certain double-stranded nucleases, particularly ribonucleases, will nick or excise RNA nucleosides from a RNA:DNA hybridization complex. Of particular use in this embodiment is RNAseH, Exo III, and reverse transcriptase.

Detailed Description Text (121):
In one embodiment, the entire scissile probe is made of RNA, the nicking is facilitated especially when carried out with a double-stranded ribonuclease, such as RNAseH or Exo III. RNA probes made entirely of RNA sequences are particularly useful because first, they can be more easily produced enzymatically, and second, they have more cleavage sites which are accessible to nicking or cleaving by a nicking agent, such as the ribonucleases. Thus, scissile probes made entirely of RNA do not rely on a scissile linkage since the scissile linkage is inherent in the probe.

Detailed Description Text (122):
In a preferred embodiment, when the scissile linkage is a nucleic acid such as RNA, the methods of the invention may be used to detect mismatches, as is generally described in U.S. Pat. Nos. 5,660,988, and WO 95/14106, hereby expressly incorporated by reference. These mismatch detection methods are based on the fact that RNAseH may

not bind to and/or cleave an RNA:DNA duplex if there are mismatches present in the sequence. Thus, in the NA.sub.1 -R-NA.sub.2 embodiments, NA.sub.1 and NA.sub.2 are non-RNA nucleic acids, preferably DNA. Preferably, the mismatch is within the RNA:DNA duplex, but in some embodiments the mismatch is present in an adjacent sequence very close to the desired sequence, close enough to affect the RNAseH (generally within one or two bases). Thus, in this embodiment, the nucleic acid scissile linkage is designed such that the sequence of the scissile linkage reflects the particular sequence to be detected, i.e. the area of the putative mismatch.

Detailed Description Text (126):
CPT may be done enzymatically or chemically. That is, in addition to RNAseH, there are several other cleaving agents which may be useful in cleaving RNA (or other nucleic acid) scissile bonds. For example, several chemical nucleases have been reported; see for example Sigman et al., Annu. Rev. Biochem. 1990, 59, 207-236; Sigman et al., Chem. Rev. 1993, 93, 2295-2316; Bashkin et al., J. Org. Chem. 1990, 55, 5125-5132; and Sigman et al., Nucleic Acids and Molecular Biology, vol. 3, F. Eckstein and D. M. J. Lilley (Eds), Springer-Verlag, Heidelberg 1989, pp. 13-27; all of which are hereby expressly incorporated by reference.

Detailed Description Text (127):
Specific RNA hydrolysis is also an active area; see for example Chin, Acc. Chem. Res. 1991, 24, 145-152; Breslow et al., Tetrahedron, 1991, 47, 2365-2376; Anslyn et al., Angew. Chem. Int. Ed. Engl., 1997, 36, 432450; and references therein, all of which are expressly incorporated by reference. Reactive phosphate centers are also of interest in developing scissile linkages, see Hendry et al., Prog. Inorg. Chem.: Bioinorganic Chem. 1990, 31, 201-258 also expressly incorporated by reference.

Detailed Description Text (128):
Current approaches to site-directed RNA hydrolysis include the conjugation of a reactive moiety capable of cleaving phosphodiester bonds to a recognition element capable of sequence-specifically hybridizing to RNA. In most cases, a metal complex is covalently attached to a DNA strand which forms a stable heteroduplex. Upon hybridization, a Lewis acid is placed in close proximity to the RNA backbone to effect hydrolysis; see Magda et al., J. Am. Chem. Soc. 1994, 116, 7439; Hall et al., Chem. Biology 1994, 1, 185-190; Bashkin et al., J. Am. Chem. Soc. 1994, 116, 5981-5982; Hall et al., Nucleic Acids Res. 1996, 24, 3522; Magda et al., J. Am. Chem. Soc. 1997, 119, 2293; and Magda et al., J. Am. Chem. Soc. 1997, 119, 6947, all of which are expressly incorporated by reference.

Detailed Description Text (129):
In a similar fashion, DNA-polyamine conjugates have been demonstrated to induce site-directed RNA strand scission; see for example, Yoshinari et al., J. Am. Chem. Soc. 1991, 113, 5899-5901; Endo et al., J. Org. Chem. 1997, 62, 846; and Barbier et al., J. Am. Chem. Soc. 1992, 114, 3511-3515, all of which are expressly incorporated by reference.

Detailed Description Text (130):
In a preferred embodiment, the scissile linkage is not necessarily RNA. For example, chemical cleavage moieties may be used to cleave basic sites in nucleic acids; see Belmont, et al.,New J. Chem. 1997, 21, 47-54; and references therein, all of which are expressly incorporated herein by reference. Similarly, photocleavable moieties, for example, using transition metals, may be used; see Moucheron, et al., Inorg. Chem. 1997, 36, 584-592, hereby expressly by reference.

Detailed Description Text (131):
Other approaches rely on chemical moieties or enzymes; see for example Keck et al., Biochemistry 1995, 34, 12029-12037; Kirk et al., Chem. Commun. 1998, in press; cleavage of G-U basepairs by metal complexes; see Biochemistry, 1992, 31, 5423-5429; diamine complexes for cleavage of RNA; Komiyama, et al., J. Org. Chem. 1997, 62, 2155-2160; and Chow et al., Chem. Rev. 1997, 97, 1489-1513, and references therein, all of which are expressly incorporated herein by reference.

Detailed Description Text (136):
Once the hybridization complex between the primary scissile probe and the target has been formed, the complex is subjected to cleavage conditions. As will be appreciated,

this depends on the composition of the scissile probe; if it is RNA, RNAseH is introduced. It should be noted that under certain circumstances, such as is generally outlined in WO 95/00666 and WO 95/00667, hereby incorporated by reference, the use of a double-stranded binding agent such as RNAseH may allow the reaction to proceed even at temperatures above the Tm of the primary probe:target hybridization complex. Accordingly, the addition of scissile probe to the target can be done either first, and then the cleavage agent or cleavage conditions introduced, or the probes may be added in the presence of the cleavage agent or conditions.

Detailed Description Text (152):
In a preferred embodiment, the detection label is a primary label. A primary label is one that can be directly detected, such as a fluorophore. In general, labels fall into three classes: a) isotopic labels, which may be radioactive or heavy isotopes; b) magnetic, electrical, thermal labels; and c) colored or luminescent dyes. Labels can also include enzymes (horseradish peroxidase, etc.) and magnetic particles. Preferred labels include chromophores or phosphors but are preferably fluorescent dyes. Suitable dyes for use in the invention include, but are not limited to, fluorescent lanthanide complexes, including those of Europium and Terbium, fluorescein, rhodamine, tetramethylrhodamine, eosin, erythrosin, coumarin, methyl-coumarins, pyrene, Malacite green, stilbene, Lucifer Yellow, Cascade Blue.TM., Texas Red, alexa dyes, phycoerythin, bodipy, and others described in the 6th Edition of the Molecular Probes Handbook by Richard P. Haugland, hereby expressly incorporated by reference.

Detailed Description Text (202):
The present invention is generally based on previous work comprising a bead-based analytic chemistry system in which beads, also termed microspheres, carrying different chemical functionalities are distributed on a substrate comprising a patterned surface of discrete sites that can bind the individual microspheres. The beads are generally put onto the substrate randomly, and thus several different methodologies can be used to "decode" the arrays. In one embodiment, unique optical signatures are incorporated into the beads, generally fluorescent dyes, that could be used to identify the chemical functionality on any particular bead. This allows the synthesis of the nucleic acids to be divorced from their placement on an array, i.e. the capture probes may be synthesized on the beads, and then the beads are randomly distributed on a patterned surface. Since the beads are first coded with an optical signature, this means that the array can later be "decoded", i.e. after the array is made, a correlation of the location of an individual site on the array with the probe at that particular site can be made. This means that the beads may be randomly distributed on the array, a fast and inexpensive process as compared to either the in situ synthesis or spotting techniques of the prior art. These methods are generally outlined in PCTs U.S. Ser. No. 98/05025 and U.S. Ser. No. 99/14387 and U.S. Ser. Nos. 08/818,199 and 09/151,877, all of which are expressly incorporated herein by reference.

Detailed Description Text (256):
As will be appreciated by those in the art, this may also be done in systems where the array is not decoded; i.e. there need not ever be a correlation of bead composition with location. In this embodiment, the beads are loaded on the array, and the assay is run. The "positives", i.e. those beads displaying a change in the optical signal as is more fully outlined below, are then "marked" to distinguish or separate them from the "negative" beads. This can be done in several ways, preferably using fiber optic arrays. In a preferred embodiment, each bead contains a fluorescent dye. After the assay and the identification of the "positives" or "active beads", light is shown down either only the positive fibers or only the negative fibers, generally in the presence of a light-activated reagent (typically dissolved oxygen). In the former case, all the active beads are photobleached. Thus, upon non-selective release of all the beads with subsequent sorting, for example using a fluorescence activated cell sorter (FACS) machine, the non-fluorescent active beads can be sorted from the fluorescent negative beads. Alternatively, when light is shown down the negative fibers, all the negatives are non-fluorescent and the the postives are fluorescent, and sorting can proceed. The characterization of the attached probe may be done directly, for example using mass spectroscopy.

Detailed Description Text (258):
Alternatively, rather than having each bead contain a fluorescent dye, each bead comprises a non-fluorescent precursor to a fluorescent dye. For example, using

photocleavable protecting groups, such as certain ortho-nitrobenzyl groups, on a fluorescent molecule, photoactivation of the fluorocherome can be done. After the assay, light is shown down again either the "positive" or the "negative" fibers, to distinquish these populations. The illuminated precursors are then chemically converted to a fluorescent dye. All the beads are then released from the array, with sorting, to form populations of fluorescent and non-fluorescent beads (either the positives and th e negatives or vice versa).

Detailed Description Text (269):
In a preferred embodiment, the DBLs may be reused by having some subpopulations of beads comprise optical signatures. In a preferred embodiment, the optical signature is generally a mixture of reporter dyes, preferably fluorescent. By varying both the composition of the mixture (i.e. the ratio of one dye to another) and the concentration of the dye (leading to differences in signal intensity), matrices of unique optical signatures may be generated. This may be done by covalently attaching the dyes to the surface of the beads, or alternatively, by entrapping the dye within the bead. The dyes may be chromophores or phosphors but are preferably fluorescent dyes, which due to their strong signals provide a good signal-to-noise ratio for decoding. Suitable dyes for use in the invention include, but are not limited to, fluorescent lanthanide complexes, including those of Europium and Terbium, fluorescein, rhodamine, tetramethylrhodamine, eosin, erythrosin, coumarin, methyl-coumarins, pyrene, Malacite green, stilbene, Lucifer Yellow, Cascade Blue.TM., Texas Red, and others described in the 6th Edition of the Molecular Probes Handbook by Richard P. Haugland, hereby expressly incorporated by reference.

Detailed Description Text (309):
Furthermore, in some embodiments, a change in the optical signature may be the basis of the optical signal. For example, the interaction of some chemical target analytes with some fluorescent dyes on the beads may alter the optical signature, thus generating a different optical signal.

CLAIMS:

12. A method according to claim 2 wherein said first target sequence is a RNA target sequence, said first primer nucleic acid is a DNA primer comprising an RNA polymerase promoter, said first enzyme is a reverse-transcriptase that extends said first primer to form a first newly synthesized DNA strand, and said method further comprises:

f) the addition of a second enzyme comprising an RNA degrading enzyme that degrades said first target sequence;

g) the addition of a third primer that hybridizes to said first newly synthesized DNA strand;

h) the addition of a third enzyme comprising a DNA polymerase that extends said third primer to form a second newly synthesized DNA strand, to form a newly synthesized DNA hybrid;

i) the addition of a fourth enzyme comprising an RNA polymerase that recognizes said RNA polymerase promoter and generates at least one newly synthesized RNA strand from said DNA hybrid; such that nucleic acid sequence-based amplification (NASBA) occurs.

14. A method for detecting a target nucleic acid sequence comprising:

a) hybridizing a first primer to a first target sequence to form a first hybridization complex;

b) contacting said first hybridization complex with a first enzyme to extend said first primer to form a first newly synthesized strand and form a nucleic acid hybrid that comprises an RNA polymerase promoter;

c) contacting said hybrid with an RNA polymerase that recognizes said RNA polymerase promoter and generates at least one newly synthesized RNA strand;

d) contacting said newly synthesized RNA strand with an array comprising:

i) a substrate with a surface comprising discrete sites; and

ii) a population of microspheres comprising at least a first subpopulation comprising a first capture probe; such that said first capture probe and the modified primer form an assay complex; wherein said microspheres are randomly distributed on said surface; and

e) detecting the presence of the newly synthesized RNA strand.

16. A method according to claim 14 wherein said target nucleic acid sequence is a RNA sequence, and prior to step a), said method comprises:

f) hybridizing a second primer comprising an RNA polymerase promoter sequence to said RNA sequence to form a second hybridization complex;

g) contacting said second hybridization complex with a second enzyme to extend said second primer to form a second newly synthesized strand and form a nucleic acid hybrid; and

h) degrading said RNA sequence to leave said second newly synthesized strand as said first target sequence.

17. A method according to claim 16 wherein said degrading is done by the addition of an RNA degrading enzyme.

18. A method according to claim 16 wherein said degrading is done by RNA degrading activity of said reverse transcriptase.

19. A method according to claim 14 wherein said target nucleic acid sequence is a DNA sequence, and prior to step a), said method comprises:

f) hybridizing a second primer comprising an RNA polymerase promoter sequence to said DNA sequence to form a second hybridization complex;

g) contacting said second hybridization complex with a second enzyme to extend said second primer to form a second newly synthesized strand and form a nucleic acid hybrid; and

h) denaturing said nucleic acid hybrid such that said second newly synthesized strand is said first target sequence.

25. A kit according to claim 21 for the detection of a NASBA reaction wherein said first enzyme is a reverse transcrptase, and said kit comprises a second enzyme that is an RNA degrading enzyme, a third primer, a third enzyme that is a DNA polymerase and a fourth enzyme that is an RNA polymerase.